

AP20 Rec'd PCT/PTO 29 JUN 2006

A PROCESS FOR THE PREPARATION AND PURIFICATION
OF RECOMBINANT PROTEINS

The present invention further relates to a novel process for the preparation and purification of viral antigenic proteins and other recombinant therapeutic proteins
5 produced in either prokaryotic or eukaryotic cell systems.

BACKGROUND OF INVENTION

Use of prokaryotic and eukaryotic cell systems for the production of various therapeutic protein molecules is a common method in present day Biotechnology. In this process, the protein of interest is expressed in the said cell system by suitably
10 engineering the molecular genetics of the expression system to incorporate a plasmid to promote the production of the desired proteins when suitably induced during the growth of the cells.

Similarly, the use of various cell substrates for the multiplication of viruses for the production of viral antigens is also a common practice. In this process, the cells are
15 multiplied to large volumes and then they are "infected" with the required virus to facilitate the growth of the viruses. Alternately, transfected cells can also be grown. The viral harvests are obtained from the culture supernates or by cell lysis.

In both the cases as above, the proteins of interest is then concentrated, purified and further treated suitably (inactivated or cleaved) to prepare a therapeutic
20 preparation or vaccine as the case may be.

The major challenges in any of the above processes are the following.

- a) Recovery of the protein or antigen of interest in a most economic way.
- b) Purification of the protein of interest to eliminate the contaminating substances like the host cell proteins, media components and any other materials used in the
25 process.

- c) Concentration of the purified protein to enable further processing.
- d) Maintenance of the functional structure and activity of the protein during various stages of purification and the efficiency of recovery.
- e) Preparation of a product of therapeutic value at the end of the process which
5 shows equal or better performance as that of the reference product.

In order to achieve the above objectives, various processes are adapted. Recombinant molecules can be expressed as heterologous proteins in yeasts such as *Sacharomyces cerevisiae*, *Pichia pastoris* or *E.coli* and other organisms. Many biopharmaceuticals and other polypeptides such as Hepatitis B, Insulin,
10 Streptokinase, Erythropoietin, Human Growth hormone have been produced by recombinant DNA technology. The expressed proteins are purified from the culture of expression host to obtain the product. Similarly several viral vaccines are also produced by culture in different types of primary or continuous cell lines. The virus grown thus is then suitably purified, concentrated and inactivated/ or used as such for
15 the preparation of vaccines.

Several steps of purification are generally adapted like clarification, centrifugation, filtration, and ultra-filtration, ammonium sulphate precipitation, use of silica beads, continuous centrifugation, rate zonal gradient centrifugation, various methods of chromatography like gel permeation, size exclusion, affinity and Ion-exchange, etc.
20 The purification processes named above have several draw backs such as multiple steps, product loss, costly equipments and consumables and some times use of harmful chemicals like Cesium chloride, etc., and some of the processes make the product non-viable due to high cost of the 'down stream process'.

BRIEF DESCRIPTION OF THE INVENTION

According to the present invention as herein described, the recombinant proteins are made to be expressed in the vectors like E.coli, yeast, Eukaryotic cell, etc., extracted and purified by using HIMAX technology. It is understood that the word 'HIMAX' is coined by the inventors and refers to only the technology developed for this invention as explained hereunder.

OBJECTS OF THE INVENTION

- 1) The first object of the invention is to provide a method for the preparation and purification of recombinant proteins from the vectors by using HIMAX technology.
- 2) The second object of the invention is to prepare recombinant proteins which are highly purified without loss of biological activity.
- 3) The third object of the invention is to achieve negligible interference of the nucleic acid or other contaminants if any during the preparation of recombinant proteins.
- 4) The fourth object of the invention is to provide a process for simultaneous concentration and purification of various recombinant proteins, viral antigens and biotherapeutic molecules.
- 5) The fifth object of the invention is to provide a process of protein purification which is less time consuming and cost effective.
- 6) Another embodiment of the invention is to provide a process of purification of live and inactivated viral antigens from cell lysate and fluid.
- 7) The seventh object of the invention is to purify the recombinant proteins by using divalent cations like Zn, Ca, Mg, etc., in combination with anions like Acetate, Phosphate and chlorides.

Accordingly the present invention relates to a process for the preparation and purification of protein(s) such as viral antigenic proteins, other recombinant therapeutic proteins characterized in that the purification is carried out by a novel technique termed as HIMAX technology which is as herein described and recovering
5 the said protein(s).

The present invention further relates to process and purification comprising:

- (a) the vector cells are subjected to lysis in the absence of a detergent to obtain a cell lysate;
- (b) subjecting the cell lysate of steps as to centrifugation ranging from 1000g to
10 10,000g;
- (c) obtaining a solid from step(b) by decantation wherein the said solid comprising the said proteins;
- (d) suspending the said solid in a buffer of pH 6 to 7.5 and optimally treating this
15 with a detergent such as herein described to solubilize the minute impurities if any;
- (e) as a part of HIMAX technology, the said protein(s) is/are captured by the addition of divalent ionic salt having concentration ranging from 0.2% to 10% with counter ions of either phosphate, chloride and/or acetate solution to form an insoluble matrix;
- 20 (f) subjecting the said insoluble matrix for centrifugation optimally to form pellets;
- (g) subjecting repeated desorptions process to release the bound antigen from insoluble matrix/pellets by using either Tris buffer of Ph 8.0 to 8.5 or Tris buffer with EDTA at Ph 7.0 to 8.0;

(h) finally recovering the said proteins through ultrafiltration, chromatography on colloidal silica, hydrophobic and or affinity chromatography, ion exchange, diafiltration, sterile filtration or a combination thereof.

- 5 The present invention further relates to process and purification of toxoids such as Diphtheria and Tetanus

DETAILED DESCRIPTION OF THE INVENTION

Now the details of the present invention:

- 10 a) The desired protein obtained through recombinant expression method or by culture in suitable tissue culture is obtained in a clarified harvest after various steps like cell lysis, cell debris removal and clarification, etc.
- b) A primary capture of the protein or antigen is carried out using the HIMAX method. Briefly the method involves using the addition of a divalent ionic salt ranging from 0.2% to 10% with counter ions of either phosphate, 15 chlorides or acetate solution to form an insoluble matrix. The insoluble matrix thus obtained is then gently centrifuged to separate the bound antigen mass. The pellet thus obtained is then desorbed repeatedly with either Tris buffer of pH 8.0 to 8.5 or Tris buffer with EDTA at pH 7.0 to 8.0.
- 20 c) The desorbate containing the desired antigen is then further processed. In case of viral antigens, the process involved could be an inactivation followed by chromatography (ion exchange). In case of other antigens the desorbate is directly taken on to chromatography purification to obtain highly pure protein.
- 25 d) The final bulk product is obtained after pooling of the chromatographically purified fractions containing the desired proteins

followed by diafiltration and

e) or sterile filtration steps.

The above steps of invention are more clearly depicted in the following examples for some recombinant and cell culture proteins.

5 The examples provided herein are only for the explanation of the invention in detail and is not to be construed that the provided examples limits the scope of the present invention.

Varying options which are within the scope of the invention but are not covered in the description that are available to the persons skilled in the art are to be taken
10 as included in the present invention.

EXAMPLE -I

Hepatitis B antigen production from a recombinant pathway.

The cell lysate after fermentation is subjected to centrifugation and the insoluble fraction is treated with detergent. The supernatant after centrifugation was either
15 subjected to Aerösil adsorption and desorption (traditional technology) (table 1) or to primary capturing of HBsAg by a batch procedure in which salts of divalent cations such as Calcium, Magnesium and Zinc are added at 0.2% to 10% (w/v) in the presence of phosphates, Chlorides or Acetates to form white insoluble matrix. The insitu formation of the matrix further interact with the antigen and this
20 process of protein capturing is referred as HIMAX technology (table 20). This matrix was separated by centrifugation between 7000g to 10,000g and bound antigen was desorbed repeatedly with this buffer of pH 8.5.

The desorbate was further purified using an anion exchange matrix namely the DEAE.

25 The HbsAg activity in all the intermediate steps is given in table I and table II.

In another strategy the cell lysate is directly subjected to primary capturing of the antigen by cations at 0.2 to 10% in the presence of phosphates, chlorides and acetates. All subsequent steps are similar to earlier procedure.

The HBs Ag activity in all the intermediate steps is given in table III.

5 **Flow Chart for HBs Ag production using HIMAX**

Large Scale Fermentation

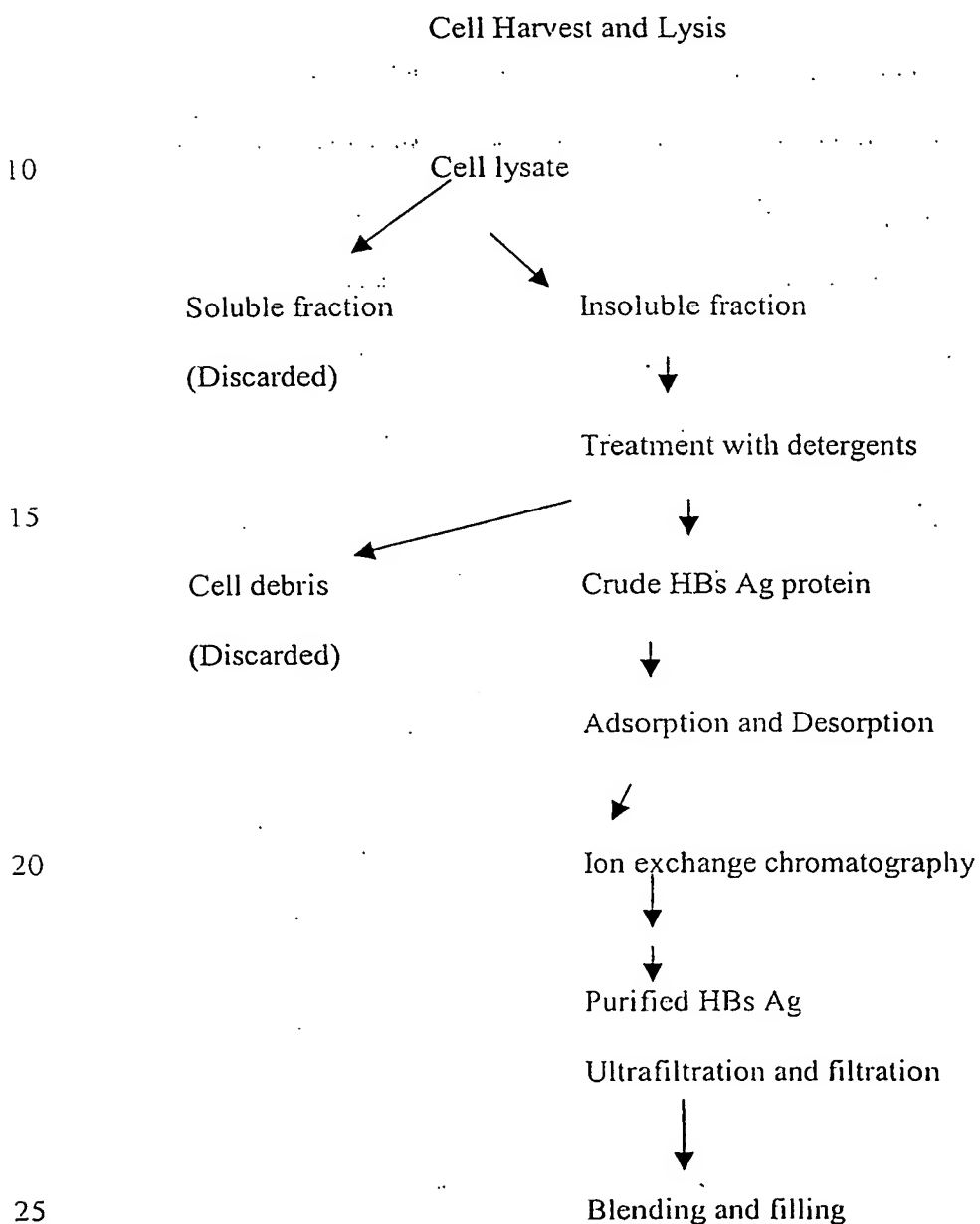


Table I Hepatitis B purification by traditional method

S. No	Purification step	Activity (%)
1.	Total cell Lysate	100
2.	Soluble fraction	9
3.	In soluble fraction (membrane bound)	91
4.	Treatment with detergent	
5.	Centrifugation	
6.	Cell debris	16
7.	Supernatant (HBsAg protein)	34
8	Binding to Aerosil and desorption	20
9	Ion exchange chromatography.	15

Table II Hepatitis B purification by HIMAX method

S. No	Purification step	Activity (%)
-------	-------------------	--------------

1.	Cell Lysate	100
2.	Soluble fraction	9
3.	In soluble fraction (HBsAg membrane bound)	91
4.	Treatment with detergent	
5.	Centrifugation	
6.	Supernatant (HBsAg protein)	84
7.	Adsorption and desorption	80
8	Ion exchange chromatography.	77

5

Table III Hepatitis B purification by HIMAX method

S. No	Purification step	Activity (%)
-------	-------------------	--------------

1.	Cell Lysate	100
2.	Adsorption and Desorption	90
3	Ion Exchange chromatography	80

The major difference between table 2 and table 3 is the usage of detergent,

In the table 2, the insoluble fraction is treated with detergent, and further processing is carried with Adsorption and desorption technology.

- 5 While in the experiments represented in table 3, the cell lysate is directly subjected to adsorption and desorption by HIMAX technology.

EXAMPLE II

Rabies antigen production from a cell culture pathway (FIG 2)

- The large scale virus culture facilitates obtaining Rabies virus in the culture supernates. Traditionally the harvests of virus thus obtained are concentrated by
 10 ultrafiltration and then purified using the gradient ultracentrifugation on sucrose in a continuous or batch mode zonal centrifuge. In the present invention the culture supernatants are initially purified by the use of HIMAX for primary capturing of rabies antigen by a batch procedure in which salts of divalent cations such as
 15 Calcium Magnesium and Zinc are added to yield a final concentration of 8 to 10 fold (W/V) resulting in the formation of white insoluble matrix further interacts. The insitu formation of the matrix further interact with the antigen and this process of

protein capturing is referred as HIMAX technology. This matrix was separated by centrifugation between 7000g to 10,000g and the bound antigen was desorbed repeatedly with tris EDTA buffer of pH 7.2.

The concentrated antigen so obtained is then inactivated by usual methods and further purified using an anion exchange matrix to obtain purified rabies antigen. The antigen is then diafiltered and blended as vaccine

The HIMAX purification yields with rabies antigen in all the intermediate steps are given in table IV.

Flow chart for HIMAX in Rabies Vaccine production

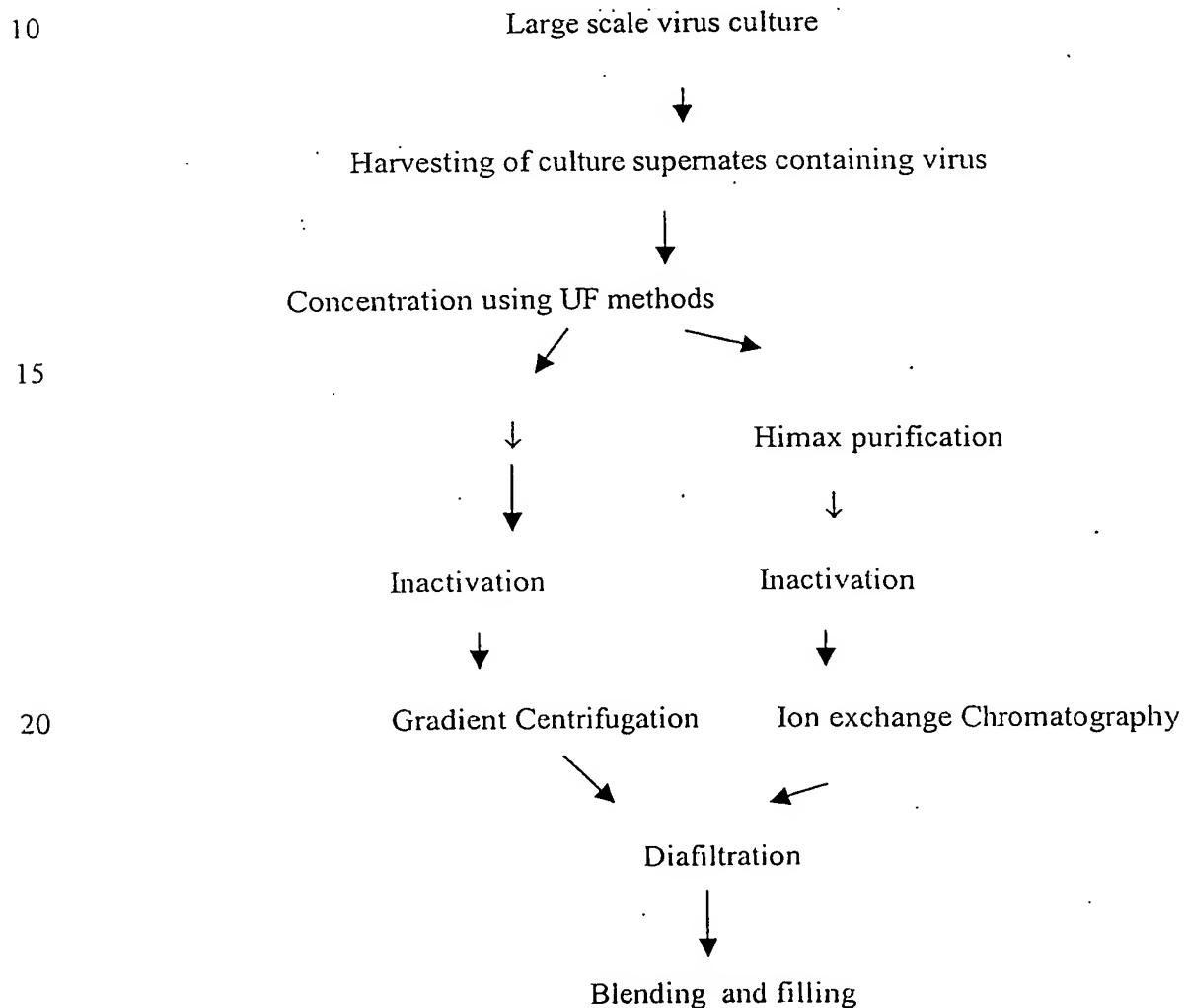


Table IV Rabies antigen purification by HIMAX

Sample lot no	Volume	HA activity per ml	Percent recovery
RAB Bulk 1-2003	1000 ml	1280	-
After HIMAX	120 ml	10240	96
RAB Bulk 2-2003	800 ml	2560	-
After HIMAX	95 ml	20480	95
RAB Bulk 3-2003	3000 ml	1280	-
After HIMAX	180 ml	20480	96

EXAMPLE III

5 Hepatitis A antigen production from a cell culture pathway:

The large scale virus culture facilitates obtaining Hepatitis A virus in the culture as cell bound virus. Traditionally the harvests of virus are obtained as cell lysates which are clarified, inactivated and then purified using the gradient ultracentrifugation on sucrose in a continuous or batch mode zonal centrifuge. In the

10 present invention the culture lysates are initially purified by the use of HIMAX for primary capturing of Hepatitis A antigen by a batch procedure in which salts of divalent cations such as Calcium Magnesium and Zinc are added to yield a final concentration of 8 to 10 fold (W/V) resulting in the formation of white insoluble matrix further interacts. The insitu formation of the matrix further interact with the

15 antigen and this process of protein capturing is referred as HIMAX technology. This

matrix was separated by centrifugation between 7000g to 10,000g and the bound antigen was desorbed repeatedly with tris EDTA buffer of pH 7.2.

The concentrated antigen so obtained is then inactivated by usual methods and further purified using an anion exchange matrix to obtain purified Hepatitis A

5 antigen. The antigen is then diafiltered and blended as vaccine

The HIMAX purification yields with Hepatitis A antigen in all the intermediate steps are given in table V.

Flow chart for HIMAX in Hepatitis A production

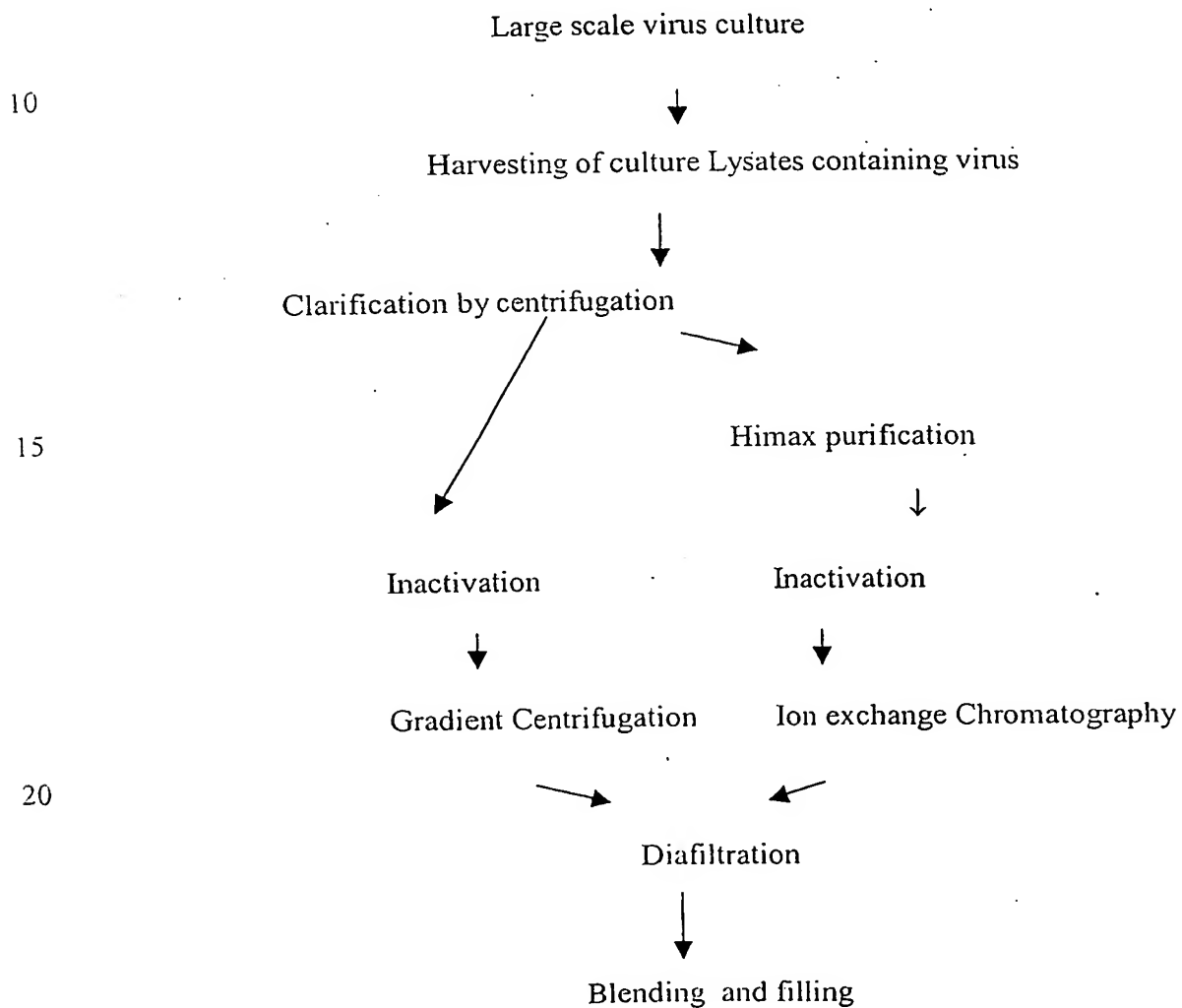


Table V -Hepatitis A antigen purification by HIMAX

Sample Lot No	Volume	ELISA units per ml	Recovery per cent
HAV Lot 2-03	100 ml	2560	
After HIMAX	9 ml	20480	72
HAV lot 3-03	150 ml	1280	
After HIMAX	16 ml	10120	84.3
HAV lot 4-03	90 ml	2560	
After HIMAX	90 ml	20480	88

Example IV:

- 5 Diphtheria toxoid is a purified protein derived from *Corynebacterium diphtheriae* Culture.

The Cell harvest is subjected to centrifugation or filtration and the toxin in the supernatant is converted to toxoid by the addition of 0.60% of formalin. The toxin is incubated at 33 C for 6 weeks for the conversion to toxoid.

- 10 The detoxification is confirmed by animal experimentation. In the traditional process the toxoid is concentrated, fractionated with Ammonium sulphate, dialysed and sterile filtered. The activity is measured by flocculation test. The recovery of toxoid is tabulated in table VI.

- In the purification by the HIMAX technology, the Toxoid is subjected to capturing,
 15 by the batch mode, in which salts of divalent cations such as Zn, Ca, Mg are added at 0.2% to 10% (w/v) in the presence of phosphates, chlorides or acetates to form white

insoluble matrix. The matrix is separated from the solution by Centrifugation between 7000 g to 10,000 g and the bound antigen is solubilized in Phosphate buffer containing 10-200 mM EDTA pH 6.8 to 7.2. The purified samples are checked by SDS-PAGE Electrophoresis.

- 5 The solution is Ultrafiltrated and the bulk is sterile filtered with 0.22micron. The results are tabulated in Table VII.

Flow Chart for Diphtheria toxoid production using HIMAX technology

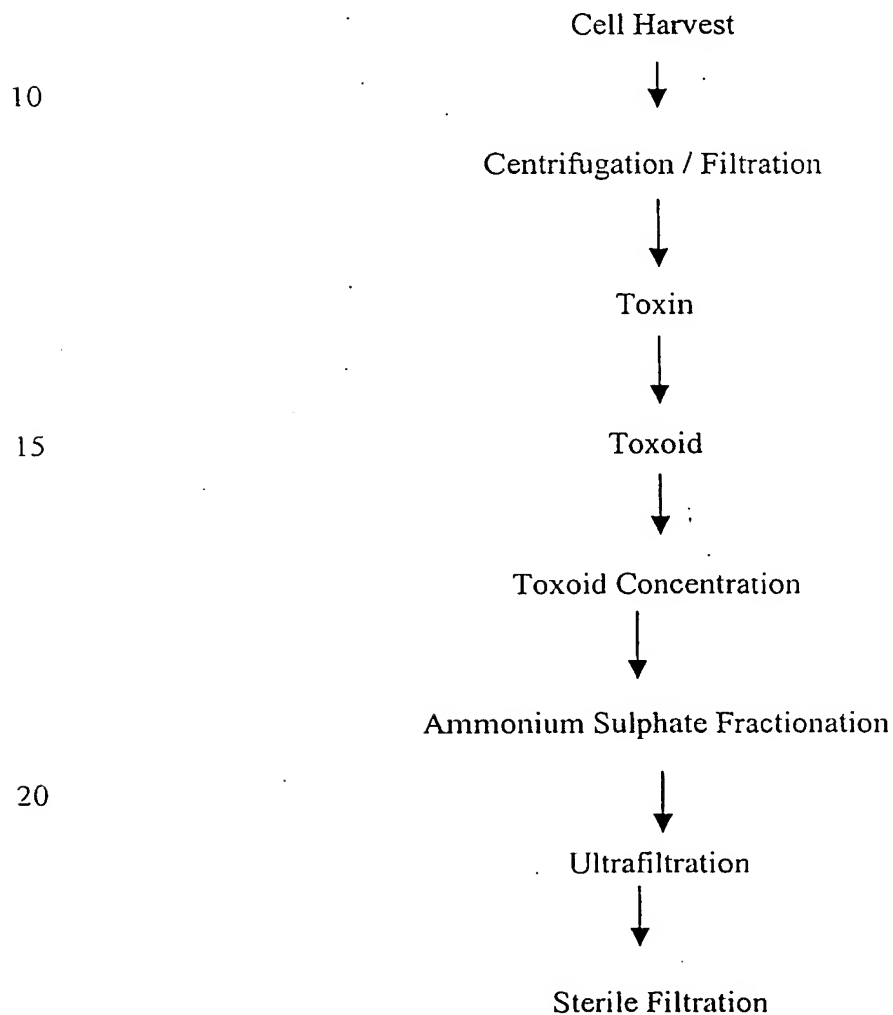


Table VI (Traditional method of purification of Ditheria toxoid)

S.No	Purification Step	Activity (%)
1.	Cell supernatant	100
2	Toxoid	90
3	Concentrated Toxoid	90
4	Ammonium Sulphate Fraction	70
5	Ultrafiltration	70
6.	Sterile filtration	70

Table VII (Purification of Diphtheria by HIMAX technology)

5

S.No	Purification Step	Activity (%)
1.	Cell supernatant	100
2	Toxoid	90
3	HIMAX purified bulk	85

4	Ultrafiltration	85
5	Sterile filtration	85

Example V:

Tetanus toxoid is a purified protein derived from *Clostridium tetani* cultures.

The Cell harvest is subjected to centrifugation or filtration and the toxin in the
 5 supernatant is converted to toxoid by the addition of 0.40% of formalin. The toxin
 is incubated at 35 C to 36 C for 4 weeks during which the toxin is converted to
 toxoid.

The detoxification is confirmed by animal experimentation. In the conventional
 process the toxoid is concentrated, fractionated with Ammonium sulphate, dialysed
 10 and sterile filtered. The activity is measured by flocculation test. The recovery of
 toxoid is tabulated in VIII

In the purification by the HIMAX technology, the Toxoid is subjected to capturing
 by the batch procedure in which salts of divalent cations such as Zn, Ca, Mg are
 added at 0.2% to 10% (w/v) in the presence of phosphates, chlorides or acetates to
 15 form white insoluble matrix. The matrix is separated from the solution by
 Centrifugation between 7000 g to 10,000 g and the bound antigen is solubilized in
 Phosphate buffer containing 10-200 mM EDTA Ph 6.8 to 7.2

The purity is checked by SDS-Electrophoresis.

The solution is Ultrafiltrated and the bulk is sterile filtered with 0.22micron. The
 20 results are tabulated in Table IX

Table VIII (Purification of Tetanus toxoid by conventional process)

S.No	Purification Step	Activity (%)
1.	Cell supernatant	100
2	Toxoid	90
3	Concentrated Toxoid	90
4	Ammonium Sulphate Fraction	70
5	Ultrafiltration	70
6.	Sterile filtration	70

Table IX (Purification of Tetanus toxoid by HIMAX technology)

S.No	Purification Step	Activity (%)
1.	Cell supernatant	100
2	Toxoid	90
3	HIMAX purified bulk	87
4	Ultrafiltration	85
5	Sterile filtration	85

5